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AFLP-RGA Markers in Comparison with RGA and AFLP in Cultivated Tetraploid Cotton

Jinfa Zhang,* Youlu Yuan, Chen Niu, Doug J. Hinchliffe, Yingzhi Lu, Shuxun Yu, Richard G. Percy, Mauricio Ulloa, and Roy G. Cantrell

ABSTRACT

Disease resistance (*R*) genes have been isolated from many plant species and R genes with domains of nucleotide binding sites (NBS) and leucine-rich repeats (LRR) represent the largest R gene family. The objective of this investigation was to test a resistance gene analog (RGA) anchored marker system, called amplified fragment length polymorphism (AFLP)-RGA in cotton (*Gossypium* spp.). The AFLP-RGA analysis uses one degenerate RGA primer designed from various NBS and LRR domains of R genes in combination with one selective AFLP primer in a PCR reaction. Out of a total of 446 AFLP-RGA bands amplified by 22 AFLP-RGA primer combinations, 76 (17.0%) and 37 (8.3%) were polymorphic within four *G. hirsutum* L. genotypes and four *G. barbadense* L. cotton genotypes, respectively. The number of polymorphic AFLP-RGA bands (256) between *G. hirsutum* and *G. barbadense* was much higher (57.4%). This level of polymorphism mirrors that of AFLP. The genetic similarity among the eight genotypes based on AFLP-RGA or AFLP lead to similar results in genotype grouping at the species and intraspecies level. However, RGA markers amplified by only degenerate RGA primers could not discriminate several genotypes. AFLP-RGA offers a great flexibility for numerous primer combinations in a genome-wide search for RGAs. Due to the distribution of RGAs or RGA clusters in the plant genome, genome-wide AFLP-RGA analysis provides a useful resource for candidate gene mapping of R genes for disease resistance in cotton.

With a better understanding of the general genome structures of higher organisms, primers derived from simple sequence repeats (SSRs), conserved regions of transposons, or retrotransposons were used in combination with random or AFLP primers to develop a number of modified marker systems such as retrotransposon–microsatellite amplified polymorphism, inter-retrotransposon amplified polymorphism, sequence specific amplification polymorphism, random amplified microsatellite polymorphism (RAMP)/digested RAMP, selective amplification of microsatellite polymorphic loci, and microsatellite-AFLP (Weising et al., 2005). Most of these markers represent random samples of the genome and have been used in various areas including genetic diversity, germplasm fingerprinting, linkage and quantitative trait locus (QTL) mapping, gene isolation, and marker-assisted selection in breeding. However, in the quest for genes responsible for evolutionary traits and plant phenotypes, functional markers from transcribed regions of the genome have recently gained more attention.

Sequence-related amplified polymorphism (SRAP) (Li and Quiros, 2001) and targeted region amplified polymorphism (TRAP) (Hu and Vick, 2003) were two recent attempts to target gene regions in a high-throughput fashion. Many sequence-tagged site (STS), cleaved amplified polymorphism, single nucleotide polymorphism, and SSR markers have also been developed from genes or expressed sequence tags in many species. In a technique recently designated single feature polymorphism (Borevitz et al., 2003), portions of gene sequences have been used as oligonucleotides for microarray hybridizations with labeled genomic DNA to simultaneously reveal genomic variations in thousands of genes. However, one of the prerequisites for single feature polymorphism is the availability of gene chips for the species of interest.

Of the many disease resistance (*R*) genes isolated in numerous plant species, R genes with domains of NBS and LRR represent the largest R gene family (Martin et al., 2003). Recent genome analyses identified approximately 150 and 500 NBS-LRR genes in *Arabidopsis* (Meyers et al., 2003) and rice (*Oryza sativa* L.) (Monosi et al., 2004), respectively. The conserved NBS domain comprising the P loop, the kinase-2 motif, and the GLPL motif has enabled the isolation of disease resistance analogs (RGAs) from numerous plant species (reviewed in Martin et al., 2003). Genetic diversity of the RGAs in relation to their origin, evolution, and germplasm diversity have been extensively investigated (Leister et al., 1996; Kanazin et al., 1996; Yu et al., 1996; Chen et al., 1998; Collins et al., 1998; Mago et al., 1999; Grube et al., 2000; Pan and Wendel, 2001; Graham et al., 2002; Rossi et al., 2003; Trognitz and Trognitz, 2005). The genetic and physical mapping of RGAs and their expression and relationships with R genes and QTL have been reported in a number of plant species (Fourmann et al., 2001; Huettel et al., 2002; Penuela et al., 2002; Quint et al., 2003; Di Gaspero and Cipriani, 2003; Hunger et al., 2003; Liu and Ekramoddoullah, 2003; Radwan et al., 2004;

J.F. Zhang, C. Niu, and Y.Z. Lu, Dep. of Plant and Environ. Sci., Box 30003, New Mexico State Univ., Las Cruces, NM 88003; Y.L. Yuan and S.X. Yu, Key Laboratory of Cotton Genetic Improvement, Ministry of Agriculture, China Cotton Research Institute, Anyang, Henan, China; D.J. Hinchliffe, USDA-ARS, Southern Regional Research Center, 1100 Robert E. Lee Blvd., New Orleans, LA 70124; R.G. Percy, U.S. Arid-Land Agricultural Research Center, USDA-ARS, 21881 N. Cardon Lane, Maricopa, AZ 85239; M. Ulloa, WCIS Res. Unit, Cotton Enhancement Program, USDA, Shafter, CA 93262; R.G. Cantrell, Cotton Inc., Cary, NC 27513. Received 15 Apr. 2006. *Corresponding author (jinzhang@nmsu.edu).

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677 S. Segoe Rd., Madison, WI 53711 USA

Abbreviations: AFLP, amplified fragment length polymorphism; GS, genetic similarities; LRR, leucine-rich repeats; NBS, nucleotide binding sites; RGA, resistance gene analog; QTL, quantitative trait locus; SRAP, sequence-related amplified polymorphism; SSRs, simple sequence repeats; STS, sequence tagged site; TRAP, targeted region amplified polymorphism.

Clement et al., 2004; Dilbirligi et al., 2004; Irigoyen et al., 2004; Rajesh et al., 2004; McIntyre et al., 2005; van Leeuwen et al., 2005; Yuksel et al., 2005).

As a DNA marker system, RGA, amplified directly from the degenerate RGA primers and revealed in polyacrylamide gels, was first proposed by Chen et al. (1998) and has been successfully used for mapping disease resistant genes (Zhang et al., 2004). However, RGAs have been usually cloned and sequenced for designing more robust STS primers in most mapping experiments (Hinchliffe et al., 2005). Hayes and Saghai Maroof (2000) proposed a modified AFLP procedure with an AFLP primer and an NBS degenerate primer in the second round of amplification to map an R gene in soybean [*Glycine max* (L.) Merr.]. Recently it also has been successfully used to isolate RGAs and map disease resistance genes in pepper (*Capsicum annuum* L.) and lupin (*Lupinus angustifolius* L.) (Egea-Gilabert et al., 2003; You et al., 2005). Soriano et al. (2005) substituted one AFLP primer by a nondegenerate RGA primer designed from nonconserved regions of the NBS domain in apricot (*Prunus armeniaca* L.) to develop and map AFLP-RGA markers. For efficient targeting of R gene loci, van der Linden et al. (2004) proposed and tested a new strategy called NBS profiling involving only one restriction enzyme (*Mse*I) and the method has been successfully used to identify RGAs and map major genes and QTL for disease resistance in apple (*Malus* spp.) (Calenge et al., 2005).

The above mentioned modified methods are almost exclusively based on the conserved NBS region. However, the LRR domains, thought to play a major role in distinct pathogen-specific recognitions, were not extensively used in RGA profiling due to their low level of sequence conservation. Here, using cultivated tetraploid cotton (*G. hirsutum* L. and *G. barbadense* L.) as an example, we have attempted to establish a RGA-anchored marker system, called AFLP-RGA by using one degenerate or nondegenerate primer designed from various regions of R genes including NBS and LRR domains in combination with one selective AFLP primer in a PCR. We demonstrate the feasibility of AFLP-RGA for the genome-wide RGA search and mapping of RGA. A number of studies have suggested that isozymes, random amplified polymorphic DNA, AFLP, restriction fragment length polymorphism, and SSR revealed a low level of polymorphism within the predominant cultivated Upland cotton (*G. hirsutum* L.) germplasm (Ulloa et al., 2005; Zhang et al., 2005a, 2005b), thus limiting the construction of genome-wide linkage map and QTL mapping in Upland cotton. The AFLP-RGA marker system should add more markers to existing cotton genetic maps and therefore be used to map other genes of interest.

MATERIALS AND METHODS

Plant Materials and DNA Isolation

To compare the resolution power among AFLP, RGA, and AFLP-RGA, eight genotypes were used, including four *G. hirsutum* cotton (TM-1, NM 24016, Acala 1517–99, and Acala Nem-X), and four *G. barbadense* cotton (SxP, Amsak, Pima 32, and Pima Phytogen 76). Among the four *G. hirsutum* cotton genotypes, TM-1 is an Upland cotton (Kohel et al., 1970), while the other three are Acala cotton with substantial germplasm introgression from *G. barbadense* cotton (Pima). Genomic DNA of each genotype from leaf tissues was extracted following the mini-prep cTAB protocol as described by Zhang and Stewart (2000).

AFLP, AFLP-RGA, and RGA Analysis

The AFLP was done following the protocol of Vos et al. (1995) with minor modifications (Zhang et al., 2005b). Briefly, genomic DNA was digested with *Eco*RI and *Mse*I and ligated with *Eco*RI and *Mse*I adaptors in the same reaction. The diluted, ligated solution was used in the first round of AFLP amplification using two preselective primers with a single selective nucleotide extension. Then, the second round of amplification was performed using the diluted preselective PCR as a template with two selective AFLP primers. For AFLP-RGA analysis, one of the degenerate RGA primers (Table 1) in combination with one of selective AFLP primers was used in the second round of PCR amplification.

PCR amplification of RGA followed the method described by Hinchliffe et al. (2005). Briefly, PCR was performed in 20-μL volumes with the following concentrations: 1× PCR buffer (10 m*M* Tris-HCl, 50 m*M* KCl, pH 8.3), 20 ng genomic DNA template, 0.2 m*M* dNTPs, 2.5 m*M* $MgCl_2$, 2 μ*M* of primers, and 0.025 U/μL of *Taq* DNA polymerase. Thermal cycling conditions were initial denaturation at 94°C for 4 min and 40 cycles at 94°C for 60 s, 43°C for 45 s, and 72°C for 90 s followed by a final extension at 72°C for 7 min. All PCR reactions in this study were performed in PE Applied Biosystems GeneAmp PCR System 9700 and/or 2720 (Applied Biosystems, Foster City, CA). The AFLP, RGA, and AFLP-RGA products were resolved in 5% polyacrylamide gels and visualized using silver staining.

Data Analysis

Fragments in the polyacrylamide gels were scored as categorical data (i.e., presence [1] and absence [0]) to form a data matrix for the eight genotypes. To estimate the genetic similarities among the eight genotypes, a genetic similarity coefficient matrix based on the Jaccard coefficient was computed for the construction of phylogenetic trees using the Neighbor–Joining (NJ) method (Saitou and Nei, 1987) of the Numerical Taxonomy System software, NTSYSpc, Version 2.1 (Exeter Software, Setauket, New York, USA).

RESULTS

Survey of Polymorphism of RGA, AFLP-RGA, and AFLP

Eight degenerate RGA primer pairs (Table 1) were tested and all amplified one to several bands as revealed by agarose gel electrophoresis (Hinchliffe et al., 2005). When the RGA products amplified by the degenerate RGA primers were separated using high resolution polyacrylamide gels, more fragments were identified. Out of 43 fragments amplified by three RGA primer pairs (NLRR-for/NLRR-rev, S2/AS3, and CLRR-for/CLRR-rev), 15 (34.9%) were polymorphic among *G. hirsutum* genotypes, while no polymorphic bands were found among the four *G. barbadense* genotypes. A total of 25 (58.1%) polymorphic markers were identified among

Table 1. Primers used to amplify putative resistance gene analogue polymorphic resistance gene analog (RGA), amplified fragment length polymorphism (AFLP), and AFLP-RGA markers.

Type	Primer	Sequence	Reference
Kinase	Pto kin-1	GCATTGGAACAAGGTGAA	Chen et al., 1998
	Pto kin-2	AGGGGGACCACCACGTAG	
	RLK-for	GAYGTNAARCCIGARAA	Feuillet et al., 1997
	RLK-rev	TCYGGYGCRATRTANCCNGGITGICC	
NBS	NBS-F1	GGAATGGGNGGNGTNGGNAARAC	Yu et al., 1996
	NBS-R1	YCTAGTTGTRAYDATDAYYYTRC	
GGBGKTT	S2	GGIGGIGTIGGIAAIACIAC	Leister et al., 1996
GLPLAL	AS3	IAGIGCIAGIGGIAGICC	
LRR	CLRR-for	TTTTCGTGTTCAACGACG	Chen et al., 1998
	CLRR-rev	TAACGTCTATCGACTTCT	
	RLRR-for	CGCAACCACTAGAGTAAC	
	RLRR-rev	ACACTGGTCCATGAGGTT	
	NLRR-for	TAGGGCCTCTTGCATCGT	
	NLRR-rev	TATAAAAAGTGCCGGACT	
	XLRR-for	CCGTTGGACAGGAAGGAG	
	XLRR-rev	CCCATAGACCGGACTGTT	
AFLP	E-ACC	GACTGCGTACCAATTCACC	
	M-CAG	GATGAGTCCTGAGTAACAG	
	E-ACC	GACTGCGTACCAATTCACC	
	M-CAT	GATGAGTCCTGAGTAACAT	
	E-ACG	GACTGCGTACCAATTCACG	
	M-CAG	GATGAGTCCTGAGTAACAG	
	E-ACG	GACTGCGTACCAATTCACG	
	M-CAT	GATGAGTCCTGAGTAACAT	
RGA-AFLP	RGA	Above 8 forward primers	
	M-CAG	GATGAGTCCTGAGTAACAG	
	RLK-for	GAYGTNAARCCIGARAA	
	M-NNN†	8 AFLP *Mse*I primers except M-CAG	
	RLK-rev	TCYGGYGCRATRTANCCNGGITGICC	
	M-CAG	GATGAGTCCTGAGTAACAG	
	E-ACG	GACTGCGTACCAATTCACG	
	RLK-for	GAYGTNAARCCIGARAA	
	E-ACG	GACTGCGTACCAATTCACG	
	RLK-rev	TCYGGYGCRATRTANCCNGGITGICC	
	PSA-E	GACTGCGTACCAATTCA	
	RLK-for	GAYGTNAARCCIGARAA	
	PSA-E	GACTGCGTACCAATTCA	
	RLK-rev	TCYGGYGCRATRTANCCNGGITGICC	
	PSA-M	GATGAGTCCTGAGTAA	
	RLK-for	GAYGTNAARCCIGARAA	
	PSA-M	GATGAGTCCTGAGTAA	
	RLK-rev	TCYGGYGCRATRTANCCNGGITGICC	

† NNN denotes CAA, CAC, CAG, CTA, CTC, CTG, or CTT.

the eight genotypes. Even though more RGA primer pairs may be tested, genome-wide coverage by RGA would be difficult, due to the nature of RGAs (i.e., targeting conserved domains of RGAs).

In comparison, 22 AFLP-RGA primer combinations (Table 1) produced a total of 446 fragments, of which 76 (17.0%) and 37 (8.3%) were polymorphic within *G. hirsutum* and *G. barbadense*, respectively. However, 256 (57.4%) AFLP-RGA markers were polymorphic between the two cotton species. When one common AFLP primer (*Eco*RI primer E-ACG) was used in combination with the RGA primers, RGA primer Pto kin-1 designed from the tomato (*Lycopersicon esculentum* Mill.) *Pto* protein kinase gene product produced 19 AFLP-RGA fragments, of which 15 were polymorphic; Due to its high degenerate nature, RGA primer RLK-for designed from wheat (*Triticum aestivum* L.) *Lr 10* gene produced the highest number of fragments (44), of which 33 were polymorphic. Surprisingly, RGA primer NBS-F1 designed from the NBS motif did not generate

Table 2. Jaccard similarity coefficients between the eight genotypes based on AFLP-RGA (amplified fragment length polymorphism–resistance gene analog), AFLP, and RGA.

Comparison		RGA	RGA-AFLP	AFLP
TM-1 vs.	NM 24016	1.000	0.879	0.868
	Acala 1517–99	0.759	0.802	0.843
	Acala Nem-X	0.622	0.773	0.738
	Pima Phy 76	0.546	0.500	0.489
	Pima 32	0.546	0.494	0.548
	SxP	0.546	0.504	0.560
	Amsak	0.546	0.503	0.560
NM 24016 vs.	Acala 1517–99	0.759	0.826	0.914
	Acala Nem-X	0.622	0.781	0.778
	Pima Phy 76	0.546	0.496	0.544
	Pima 32	0.546	0.472	0.627
	SxP	0.546	0.478	0.639
	Amsak	0.546	0.481	0.525
Acala 1517–99 vs.	Acala Nem-X	0.735	0.917	0.848
	Pima Phy 76	0.613	0.528	0.532
	Pima 32	0.613	0.509	0.631
	SxP	0.613	0.519	0.643
	Amsak	0.613	0.518	0.512
Acala Nem-X vs.	Pima Phy 76	0.475	0.550	0.588
	Pima 32	0.475	0.527	0.581
	SxP	0.475	0.534	0.591
	Amsak	0.475	0.528	0.457
Pima Phy 76 vs.	Pima 32	1.000	0.932	0.813
	SxP	1.000	0.898	0.825
	Amsak	1.000	0.898	0.737
Pima 32 vs.	SxP	1.000	0.944	0.986
	Amsak	1.000	0.943	0.812
SxP vs.	Amsak	1.000	0.985	0.800

any polymorphic AFLP-RGA fragments. However, RGA primers designed from LRR regions produced 7 to 24 ($\mu = 17.4$) AFLP-RGA fragments, of which 4 to 12 ($\mu = 8.2$) were polymorphic.

As a comparison, four AFLP primer pairs amplified 92 fragments (23 fragments per primer combination), of which 21 (22.8%) and 22 (23.9%) were found to be polymorphic within *G. hirsutum* and *G. barbadense*, respectively. The number of polymorphic AFLP markers at the species level was 57 (62.0%).

Genetic Similarities Revealed by AFLP-RGA

Jaccard similarity coefficients were used to estimate genetic similarities (GS) between the eight genotypes based on AFLP-RGA, AFLP, and RGA markers (Table 2). The GS measured by AFLP-RGA are highly correlated with those measured by AFLP and RGA ($r = 0.896$ and $r = 0.994$, respectively, $n = 28$, $P < 0.01$), however, the GS determined by AFLP is not as highly correlated with the GS measured by RGA ($r = 0.804$, $n = 28$, $P < 0.01$). This was evident in the inability to separate TM-1 from NM 24016 and to differentiate the four *G. barbadense* genotypes.

Cluster Analysis

Cluster analyses using AFLP-RGA, AFLP, or RGA markers were equally successful in dividing cultivars of the *G. hirsutum* and *G. barbadense* species into two distinct groups (Fig. 1–3). Within the *G. barbadense* subgroup, the greatest agreement between cluster analyses and known pedigrees occurred using the AFLP-RGA markers (Fig. 1). The cultivar SxP was developed from a cross of the Sakel and Pima cultivars in 1935 (Smith et al., 1999). Amsak was derived (1946) from backcrossing SxP to its Sakel parent. Given that Amsak and SxP share common parents (in different dosages) one would expect these lines to cluster together as in the AFLP-RGA dendrogram. The cultivar Pima 32 derives from the cross SxP × Pima × Giza-7, and therefore shares the Pima and Sakel parentage of SxP and Amsak. However, Pima 32 also has the Egyptian cultivar Giza-7 as a parent—which is reflected in its separation from SxP and Amsak in the AFLP-RGA dendrogram. Pima 32, SxP, and Amsak are all "pure" *G. barbadense* genotypes; whereas, Phytogen 76 is derived from a hybrid germplasm pool, created in 1948, which included introgression from *G. hirsutum*. Almost all modern American Pima cultivars

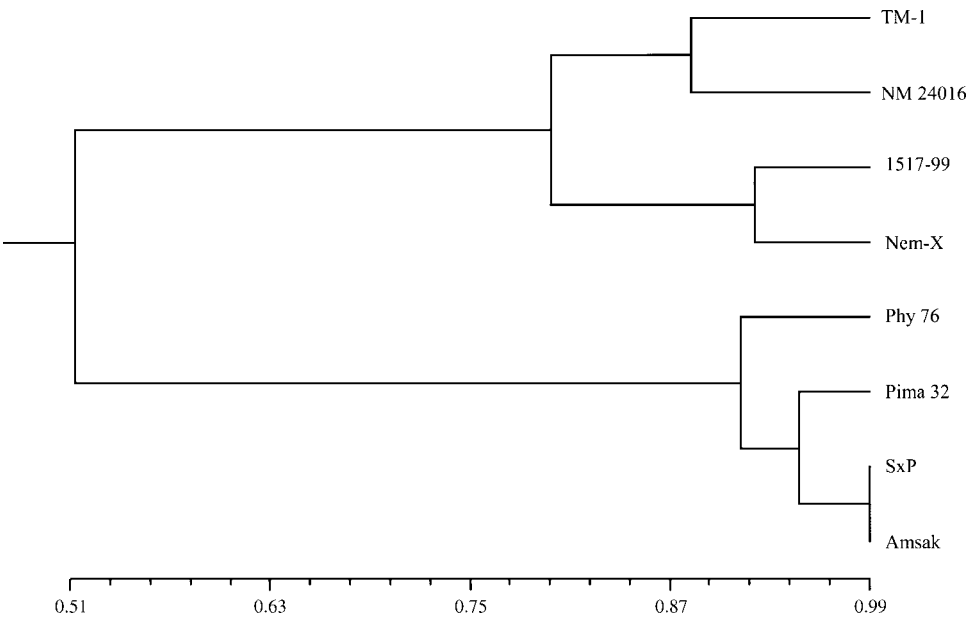

Fig. 1. An Unweighted Pair Group Method with Arithmetic Average (UPGMA) dendrogram based on AFLP-RGA markers.

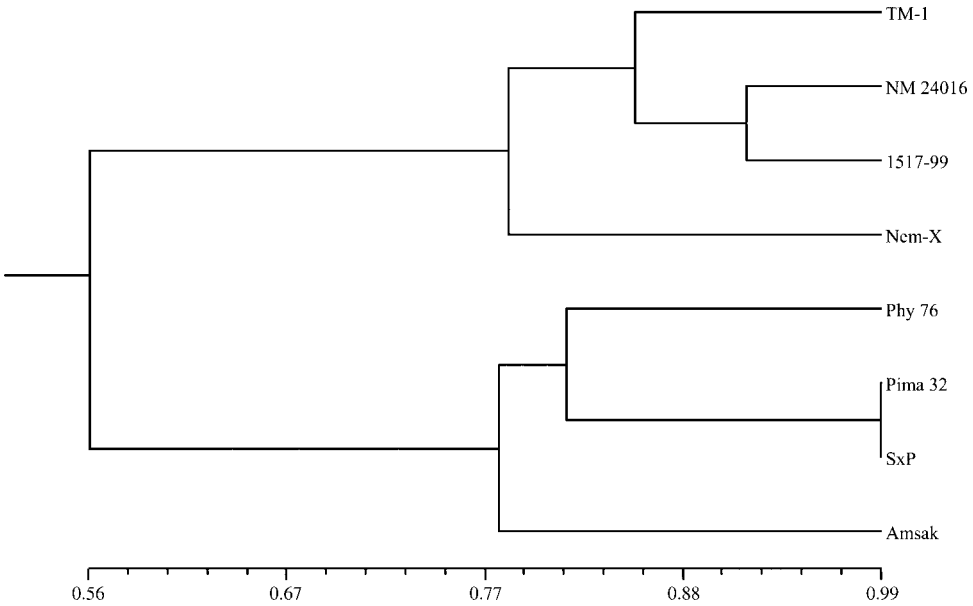

Fig. 2. An Unweighted Pair Group Method with Arithmetic Average (UPGMA) dendrogram based on AFLP markers.

derive from this hybrid pool (Smith et al., 1999). Specifically, Phytogen 76 was developed from Phy P625, a reselection from P53 and H417, a reselection from Pima S-6 (Dr. Joe Mahill, PhytoGen Cottonseeds, personal communication, 2006). The AFLP-RGA dendrogram separated Phytogen 76 from the other Pima cultivars, perhaps reflecting its hybrid origins. The AFLP dendrogram of the Pima subgroup (Fig. 2) was less informative with regards to the *G. barbadense* breeding history. Low levels of RGA polymorphism could not separate the four *G. barbadense* genotypes (Fig. 3).

Although AFLP appears to generate more polymorphism within the two cultivated species than AFLP-RGA that should render a higher resolution power in genotype separation, a larger number of primer combinations used in the AFLP-RGA analysis yielded more polymorphic bands to be utilized in the cluster analysis. Therefore, due perhaps to the limited numbers of primer combinations, AFLP and RGA cluster analyses were also less successful in reflecting known breeding history among the four *G. hirsutum* cultivars. Acala 1517–99 and Acala Nem-X originate from a common germplasm pool (Smith et al., 1999), and this relationship is reflected in the AFLP-RGA dendrogram (Fig. 1). However, in the AFLP and RGA dedrograms (Fig. 2 and 3) Acala Nem-X did not cluster with Acala 1517–99 and it was unexpectedly distant from the other three *G. hirsutum* cultivars. Also unexpected was the clustering of TM-1 with NM 24016 in the AFLP-RGA and RGA dendrograms. TM-1 has been as a standard representa-

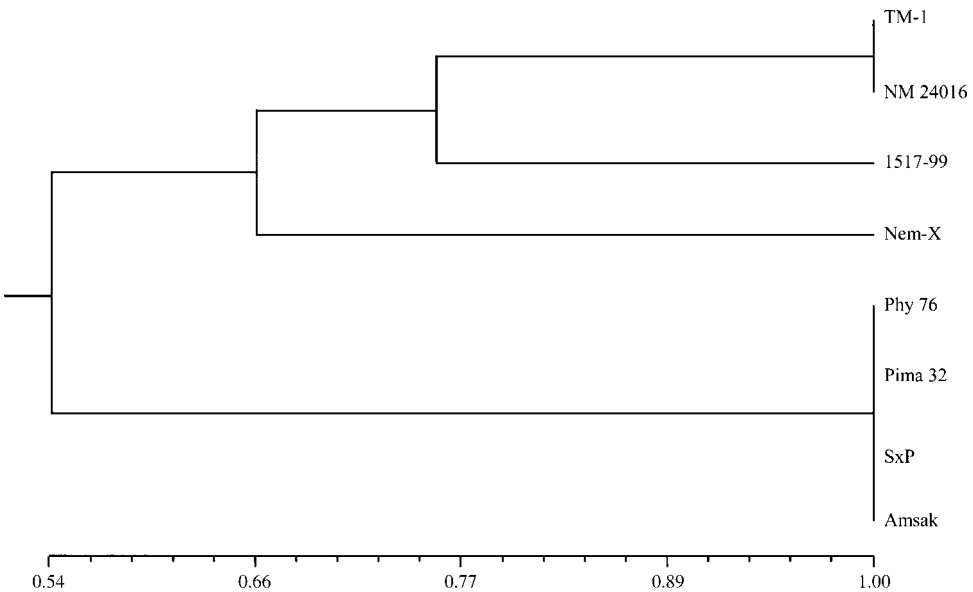

Fig. 3. An Unweighted Pair Group Method with Arithmetic Average (UPGMA) dendrogram based on RGA markers.

tive of *G. hirsutum* genome, whereas NM 24016 is the product of a recent interspecific hybridization and is considered to be highly introgressed with *G. barbadense* (Cantrell and Davis, 2000; Percy et al., 2006). Despite their breeding histories, TM-1 and NM 24016 grouped together due to the lack of RGA polymorphism between them.

DISCUSSION

In the present study, three RGA primer pairs, four AFLP primer pairs, and 22 AFLP-RGA primer pairs were used to amplify polymorphic markers among eight tetraploid cotton genotypes (Table 1). RGA markers amplified by the degenerate RGA primers generated a low level of polymorphism that could not discriminate several genotypes, especially closely related genotypes (Fig. 3). The decreased polymorphism for RGA may have resulted from using a limited number of RGA primer pairs and its nature of targeting gene regions. Even though using more RGA primer pairs should produce more polymorphic RGA markers, the low polymorphism level of this marker system is mainly related to the fact that the RGA primers are designed based on conserved R-gene coding regions. In another study using all possible eight pairs of RGA primer combinations, only 88 polymorphic RGA markers were produced in a interspecific population between NM 24016 (*G. hirsutum*) and Pima 3–79 (*G. barbadense*) (Niu et al., unpublished, 2006). It is apparent that this level of polymorphism will limit the usefulness of a genome-wide search and mapping for RGAs. On the other hand, the polymorphism level of AFLP markers appeared to be higher than both RGA and AFLP-RGA marker systems; however, this was not sufficient to discriminate among all 8 genotypes (Fig. 3). More AFLP primer combinations could increase the resolution power of AFLP in genotype discriminations. However, in a more comprehensive study, we surveyed polymorphic level of AFLP between TM-1 and NM 24016 and found only 4.5% polymorphic AFLP markers in a total of 4679 fragments amplified by 64 AFLP primer combinations and resolved using capillary CEQ 8000 DNA Sequencer (Beckman Coulter, Inc., Fullerton, CA) (Zhang et al., 2005b). In the present study, 32 (7.2%) polymorphic AFLP-RGA markers were identified between TM-1 and NM 24016. Out of a total of 446 AFLP-RGA bands amplified by 22 AFLP-RGA primer combinations, 76 (17.0%) and 37 (8.3%) were polymorphic within four *G. hirsutum* genotypes and four *G. barbadense* genotypes, respectively. The number of polymorphic AFLP-RGA markers (256) at the interspecific level was much higher (57.4%) than the intraspecific level. The genetic similarity between the eight genotypes based on AFLP-RGA was high correlated with that measured by AFLP, leading to similar results in genotype grouping at the inter-species and intra-species level.

Our data showed that AFLP-RGA and AFLP each generated on average 12 polymorphic markers per primer combination between *G. hirsutum* and *G. barbadense* genotypes. Thus, in a segregating population made between the two cultivated cotton species, approximately 1200 AFLP-RGA polymorphic markers from about 100 AFLP-RGA primer combinations could be expected and mapped. However, the number may be overestimated because the data result from a comparison between the two species from four genotypes each. In mapping, only two genotypes are considered and therefore the expected number of informative markers will be reduced.

A portion of the AFLP-RGA markers, 10 to 80% depending on primer specificity, might not be related to RGA (Hayes and Saghai Maroof, 2000; van der Linden et al., 2004; Xiao et al., 2006). Higher level of RGA-related AFLP-RGA markers could be obtained using more RGA specific primers and high annealing temperatures. Nevertheless, the AFLP-RGA offers many advantages in combining the high-throughput approach of AFLP with gene-anchored amplification and can provide more markers that are possibly distributed in other regions of the genome, thereby increasing genome coverage. By contrast, NBS profiling protocol was specifically designed for RGA-NBS anchored amplification only and cannot be used for AFLP analysis (van der Linden et al., 2004). Previous reports focused on the NBS domain, which is more conserved than the LRR domains, therefore decreasing the level of polymorphism. Since the LRR domain is more variable and determines pathogen specificity, its polymorphism should be more important in determining the genetic variation of disease resistance in R gene mapping. Keeping this in mind, we used both degenerate NBS and LRR primers in combination with one selective AFLP primer and have successfully developed AFLP-RGA markers. The number of total and polymorphic AFLP-RGA markers may depend on primer combinations and degeneracy of the RGA primers. It should be noted that LRR is a widespread protein–protein interaction motif and found in a functionally diverse array of proteins encoded by many genes including NBS-LRR R genes. It seems therefore more than likely that a significant portion of LRRs from other gene families could be amplified by AFLP-RGA. Only sequencing can resolve the question that the majority of the fragments amplified with the applied AFLP-RGA markers are indeed located within R genes.

In most mapping studies, RGAs amplified by degenerate primers were cloned and sequenced to develop more robust RGA-STS markers. However, the level of polymorphism is limited. For example, in our previous study (Hinchliffe et al., 2005), out of 61 RGA-STS primer pairs only nine (7.0%) amplified polymorphic RGA-STS markers between *G. hirsutum* (NM 24016) and *G. barbadense* (3–79) when resolved in the agarose gels. However, RGA markers detected by single strand conformational polymorphism (RGA-SSCP) were shown to be more polymorphic (Kuhn et al., 2003). AFLP-RGA is a gene-targeted functional marker system. It has the high throughput feature of AFLP, SRAP, and TRAP markers but is less problematic (due to higher annealing temperatures used for AFLP-RGA) than SRAP and TRAP because of less mismatch between primer sequences and template DNA targets. Even though the RGA primers are degenerate, degeneracy allows more

template sites for primer annealing and does not increase their mismatch with DNA template. In the TRAP marker system, Hu et al. (2005) reported that only 1% of the cloned fragments amplified by a gene primer and a random primer were from the targeted expressed sequence tags. However, TRAPs were found to be highly repeatable and efficient in generating hundreds of markers for mapping in wheat (Liu et al., 2005).

The reliability of AFLP-RGA can be assessed by (i) the repeatability using same DNA in same PCR conditions with same primers but in different PCR runs, or (ii) the proportion of common fragments amplified within the same species, especially closely related genotypes, as reflected by genetic similarities (GS) among them and their dendrogram. The average GS within *G. hirsutum* and *G. barbadense* was 83.0 and 93.3%, respectively, and 50.9% between the two cultivated species. The results are congruent with the evolutionary and breeding history of the two species and data obtained from other marker systems (Zhang et al., 2005a, 2005b). Furthermore, the high proportion of common AFLP-RGA fragments (94.4–98.5%) were shared among the three closely related early Pima cultivars (SxP, Amask, and Pima 32). This again demonstrates the reliability of the AFLP-RGA marker system. The expression and polymorphism of AFLP-RGA at the transcription level should be investigated using cDNA.

From the knowledge about the *Arabidopsis* and rice genome sequences, each chromosome contains NBS-LRR genes, some of which are clustered. On most chromosomes it appears that on average every 5 to 20 cM contains an RGA or RGA cluster (Meyers et al., 2003; Monosi et al., 2004). Therefore, AFLP-RGA could be used as chromosome anchored markers for disease resistance candidate gene mapping. Furthermore, many RGA may also be in the proximity with other genes or in gene-rich regions. Therefore, polymorphic AFLP-RGA markers can be also used for mapping other traits. Degenerate RGA primers in combination with selective AFLP primers can provide numerous AFLP-RGA markers to conduct genome-wide mapping of R genes and RGA in any plant species in which no prior sequence information is required.

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